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ORIGINAL PAPER

Mycogenesis of gold nanoparticles using a phytopathogen *Alternaria alternata*

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Abstract The development of an eco-friendly and reliable process for the synthesis of gold nanomaterials (AuNPs) using microorganisms is gaining importance in the field of nanotechnology. In the present study, AuNPs have been synthesized by bio-reduction of chloroauric acid ($HAuCl_4$) using the fungal culture filtrate (FCF) of *Alternaria alternata*. The synthesis of the AuNPs was monitored by UV–visible spectroscopy. The particles thereby obtained were characterized by UV, dynamic light scattering (DLS), X-ray diffraction (XRD), energy dispersive X-ray (EDX) analysis, Fourier transform infrared (FTIR) spectroscopy, atomic force microscopy (AFM) and transmission electron microscopy (TEM). Energy-dispersive X-ray study revealed the presence of gold in the nanoparticles. Fourier transform infrared spectroscopy confirmed the presence of a protein shell outside the nanoparticles which in turn also support their stabilization. Treatment of the fungal culture filtrate with aqueous Au^+ ions produced AuNPs with an average particle size of 12 ± 5 nm. This proposed mechanistic principal might serve as a set of design rule for the synthesis of nanostructures with desired architecture and can be amenable for the large scale commercial production and technical applications.

Keywords *Alternaria alternata* · Fungal culture filtrate · Gold nano

Abbreviations

FCF	Fungal culture filtrate
DLS	Dynamic light scattering
XRD	X-ray diffraction
EDX	Energy dispersive X-ray
FTIR	Fourier transform infrared
AFM	Atomic force microscopy
TEM	Transmission electron microscopy
$HAuCl_4$	Chloroauric acid
AuNPs	Gold nanoparticles

Introduction

Nanotechnology, the powerful tool for the creation of new objects in nanoscale dimensions, is a cutting edge technology having important applications in modern biomedical research [1–4]. As the dimension of nanoscale devices is similar to cellular components such as DNA and proteins [5, 6], the tools developed through nanotechnology may utilize AuNPs to detect several diseases at the molecular level [7]. But in the past few years, researchers have began to realize the possible toxicities of AuNPs which are to some extent traced back to the chemical ingredient used in conventional method [8]. Conventional synthetic methods of AuNPs have involved a number of chemical methods [9, 10]. With the rapid development of these chemical

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methods, concern for environmental contaminations is regularly heightened as the chemical procedures involved in the synthesis of nanomaterials generates a large amount of hazardous by-products. Thus, there is an urgent need for 'green chemistry' that includes clean, nontoxic and environment-friendly methods of nanoparticle synthesis with precise control over the shape and size. In the recent years, 'green synthesis' of the nanoparticles has paid much more attention in the rapidly growing area of nanoscience and nanotechnology [11–15].

Biological systems have been widely utilized to synthesize and assemble a range of inorganic nanomaterials from bacteria [16], algae [17] and fungi [18]. Many microbes are known to produce highly structured metallic nanoparticles with very similar properties to that of chemically synthesized materials, while having precise control over size, shape and monodispersity [19]. Filamentous fungi in general possess some distinctive advantage over bacteria and algae, because of their high metal tolerance and bioaccumulation ability [20]. Additional benefits of using fungi in nanoparticle synthesis are ease in their scale-up, downstream processing, handling of biomass and economic viability. Besides these, fungi are extremely efficient secretor of extracellular enzymes; it is thus possible to easily obtain large-scale production of enzymes. Literature survey showed a number of fungi to produce nanoparticles either intracellularly or extracellularly [18, 21–23]. Gold nanoparticles are of interest, mainly due to their stability under atmospheric conditions, resistance to oxidation and biocompatibility [24–27]. These unique properties can potentially be exploited in a diverse range of industrial applications using their optical and electronic properties in optics, electronics, medical diagnostic and treatments, senders and coating [24, 26, 28]. Earlier attempts have been made for extracellular biosynthesis of AuNPs using different filamentous fungi like *Verticillium* sp. [29], *Fusarium oxysporum* [30], *Aspergillus flavus* [31], *Colletotrichum* sp. [32], *Aspergillus clavatus* [19], *Trichothecium* sp. [33].

In this context, we present the single step 'green synthesis' protocol for the production of AuNPs utilizing culture filtrate of a phytopathogenic fungus *Alternaria alternata* (strain number: MAMP/C/51).

Materials and methods

Isolation of the pathogen and culture maintenance

The pathogen, *A. alternata* (strain number: MAMP/C/51) isolated previously by Maiti et al. [34] was grown aerobically in 50 ml of fully autoclaved potato-dextrose broth (HiMedia Laboratories Pvt. Ltd., Mumbai, India) in Erlenmeyer flasks having the capacity of 250 ml and kept for 12 days at 30 °C temperature. After the incubation period, the media was filtered with Whatman filter paper No. 1. 100 ml of that fungal culture filtrate (FCF) was taken in a sterilized Erlenmeyer flask which was further used for the reduction of the chloroauric acid aqueous solution.

Synthesis of gold nanoparticles

The chemical chloroauric acid ($HAuCl_4$) was purchased from Sigma, St. Louis, MO, USA. 100 ml of FCF was taken in each different Erlenmeyer flask and mixed with chloroauric acid solution (3 mM final concentration). The FCF containing flask was agitated for 24 h at room temperature. Simultaneously, only the culture filtrate of *A. alternata* and only chloroauric acid solution were maintained under same conditions.

The gold nanoparticles were separated out by centrifugation at 12,000*g* for 10 min, and the settled nanoparticles were washed with deionized water (three times). The purified AuNPs were resuspended in deionized water and ultrasonicated by Piezo-u-sonic ultrasonic cleaner (Pus-60w). Synthesis of AuNPs was repeated for three times ($n = 3$) and subsequently utilized for characterization of the particles.

UV–visible spectroscopy

The absorption at 540 nm (maximum absorbance observed in the full range scan) was measured continuously to determine the stability of the solution. The UV–visible spectra of the solution were kinetically monitored in the range of 200–900 nm recorded in a Hitachi 330 spectrophotometer. Deionized water was used as blank.

Size measurement by dynamic light scattering (DLS) experiment

Particle size was measured by laser diffractometry using a nano size particle analyzer (Zen 1600 Malvern USA) in the range between 0.6 nm and 6.0 μm, under the following conditions: particle refractive index 1.590, particle absorption coefficient 0.01, water refractive index 1.33 and temperature 25 °C.

X-ray diffraction (XRD) analysis

After bio-reduction, the liquid reaction mixture was dried at 45 °C in a vacuum drying oven. The vacuum-dried mixture was then collected and used for powder XRD analysis. The spectra were recorded in a PW. 3040/60 PANalytical X-ray diffractometer (Cu Kα radiation,

λ 1.54443) running at 45 kV and 30 mA. The diffracted intensities were recorded from 30° to 90° 2θ angles.

Energy dispersive X-ray (EDX) analysis

Energy-dispersive X-ray (EDX) analysis was carried out by the Hitachi S 3400 N instrument (Japan) and employed to know the elemental compositions of the particles. Samples were filtered and dried before measurements.

Fourier transform infrared spectroscopic (FTIR) analysis

For FTIR analysis, the vacuum-dried culture filtrate and AuNPs and culture filtrate were mixed separately with potassium bromide (KBr) at a ratio of 1:100 and the spectra were recorded with a Shimadzu 8400S fourier transform infrared spectrophotometer using a diffuse reflectance accessory. The scanning data were obtained from the average of 50 scans in the range between 4,000 and 400 cm^{-1}.

Atomic force microscopic (AFM) observation of gold nanoparticles

The size and surface topography of the drop coated film of the AuNPs was investigated with atomic force microscope [Nanoscope (R) 111a Veeco multimode, USA] and high resolution surface images were produced. In AFM characterization, the tapping mode with RTESP tip was used. The resonance frequency was 287.64 and the scanning range was 1.25 μm.

Transmission electron microscopic (TEM) observation of gold nanoparticles

TEM samples of the aqueous suspension of AuNPs were prepared by placing a drop of the suspension on carbon-coated copper grids and allowing the water to evaporate. The micrographs were obtained by Tecnai G^2 spirit Biotwin (FP 5018/40) TEM, operated at 80 kV accelerating voltage.

Results and discussion

Production and characterization of gold nanoparticles

The culture filtrate mediated synthesis of AuNPs was validated by visually monitoring the three flasks containing only the culture filtrate of *A. alternata*, reaction mixture of the culture filtrate with chloroauric acid and only chloroauric acid solution. Only the reaction mixture displayed a time dependent color change, whereas the fungal culture filtrate and the chloroauric acid solution were observed to retain their original color (Fig. 1 inset). At the beginning, the reaction mixture was light yellow and became pinkish after 1 h of incubation at room temperature; the pinkish color changed to dark red gradually at 24 h and then the red color did not change with increasing incubation time. The appearance of the red color indicated the occurrence of the reaction and the formation of Au nanoparticles.

UV–visible spectroscopic analysis

The characteristic red color of the reaction solution was due to the excitation of the surface plasmon vibration of Au^0 particles and provided a convenient spectroscopic signature of their formation. Both the control set showed no significant color change in the same experimental conditions. The reduction of chloroauric acid was subjected to spectral analysis by using the UV–visible spectrophotometer. This showed an absorbance peak at 540 nm (Fig. 1), which was specific for gold nanoparticles [19].

Particle size measurement

Particle size was determined by dynamic light scattering measurement. Laser diffraction revealed that particle size obtained in the range of 2–30 nm (Fig. 2).

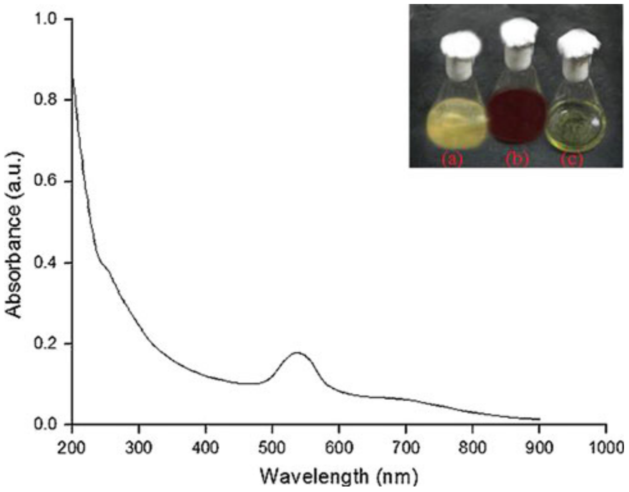

Fig. 1 UV–visible spectra recorded as a function of reaction time of an aqueous solution of 3 mM $HAuCl_4$ with the fungal culture filtrate (FCF). Inset: Represents three flasks containing only the culture filtrate of *Alternaria alternata* (**a**), reaction mixture of the culture filtrate with chloroauric acid (**b**) and only chloroauric acid solution (**c**). The appearance of red color only in the flask (**b**) due to the addition of *Alternaria alternata* FCF to 3×10^{-3} M aqueous $HAuCl_4$ solution after 24 h incubation period

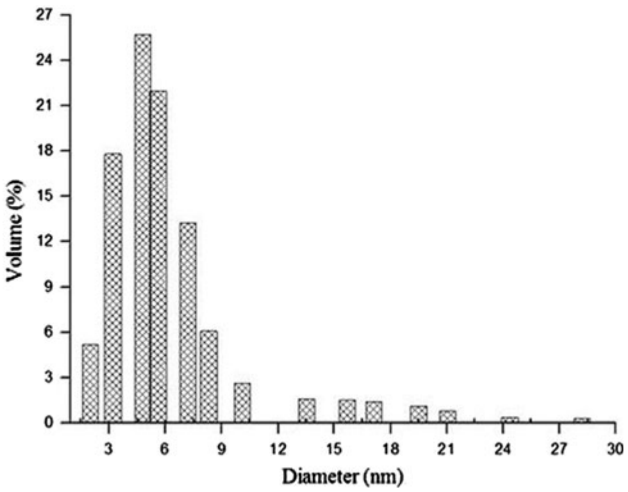

Fig. 2 Histogram of particle size distribution as obtained from light scattering of the gold nanoparticles produced by *Alternaria alternata*

XRD analysis

In order to confirm the mono-crystalline nature of the AuNPs, XRD analysis was performed. Figure 3 shows the X-ray diffraction pattern obtained from the AuNPs. The Bragg reflections obtained from the AuNPs clearly correspond to the fcc crystalline structure of gold. The XRD pattern exhibited four identical diffraction peaks corresponding to the [111], [200], [220] and [311] appearing at $2\theta = 38.2°$, 44.5°, 65.6° and 78.6° of metal gold, respectively (International Center for Diffraction Data, ICDD No. 04–0783), indicating that the precipitate was composed of pure crystalline gold [19]. As per the XRD pattern, a very intense Bragg reflection for the [111] lattice was observed, suggesting that the [111] oriented AuNPs were lying flat [19] on the planar surface, while the reflections correspond to [220] and [311] with lattice spacing of 1.44 and 1.23 Å was specific for the triangular morphology, respectively. It was also notable that the ratio of intensity between the [200] and [111] diffraction peaks for the prepared sample was much lower than the standard (0.042 vs. 0.33), and this rationally decreased as the particles size increased. These observations confirmed that as-formed AuNPs were primarily dominated by [111] facets [19], which were quite consistent with the above electron diffraction observation.

EDX observation of gold nanoparticles

An elemental composition analysis employing EDX showed the presence of a strong signal from gold atoms (Fig. 4). Moreover, the presence of sharp optical absorption peak in the range of 2–3 keV which is typical for the absorption of metallic gold nanocrystallites [35]. This analysis indicated that the nanostructures were composed solely of gold. However, there were other EDX peaks for C, O, K and N, suggesting that they were mixed precipitates from the culture filtrate.

FTIR analysis of gold nanoparticles

FTIR absorption spectra of vacuum dried fungal culture filtrate and reduced chloroauric acid by culture filtrate are shown in Fig. 5a, b, respectively. Both the spectra showed the presence of bonds due to O–H stretching (around $\sim$3,430 cm^{-1}) and aldehydic C–H stretching (around $\sim$2,920 cm^{-1}) [36]. These peaks indicated the presence of proteins and other organic residues, which might have produced extracellularly during the growth of *A. alternata*.

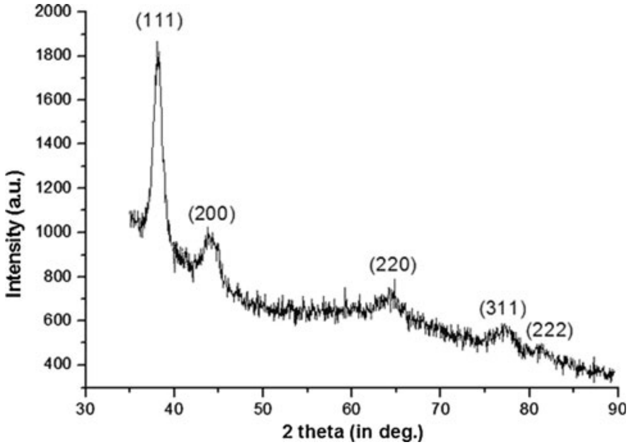

Fig. 3 Representative XRD patterns of gold nanoparticles synthesized by the reaction of 3 mM aqueous $HAuCl_4$ solution with *Alternaria alternata* culture filtrate

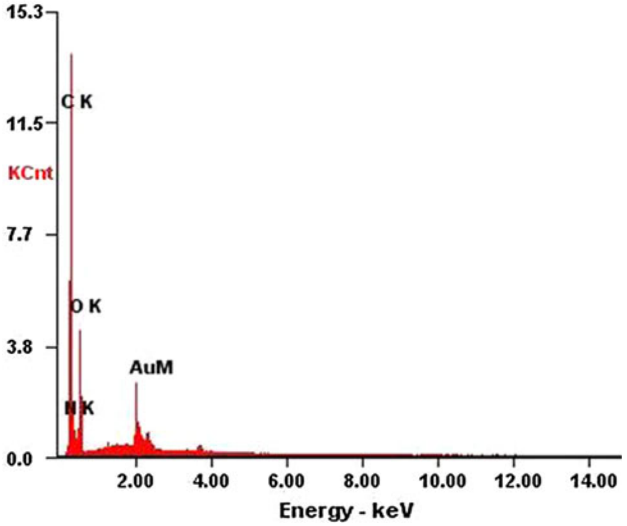

Fig. 4 EDX spectrum recorded showing sharp peak in between 2 and 3 keV confirming the presence of gold

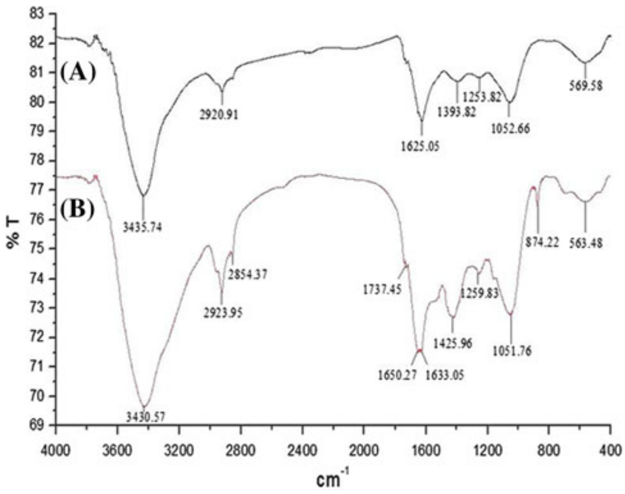

Fig. 5 FTIR absorption spectra of FCF after (**a**) and before (**b**) bioreduction of chloroauric acid

FTIR spectrum of fungal culture filtrate showed absorption band at 1,625 cm^{-1}, corresponding to the amide I of polypeptides [36]. Following reaction with $HAuCl_4$ solution, the amide I band appeared at 1,633 and 1,650 cm^{-1} [36]. The intense band found in Fig. 5b at 1,425 and 874 cm^{-1} corresponding to the C–H in plane deformation with aromatic ring stretching [37, 38]. The FTIR peak at around 1,240–1,260 cm^{-1} present in both the cases signified amide III band of the random coil of protein [39]. Band at 1,052 cm^{-1} in both the spectra indicated C–O stretching [37]. The bands visible in between 500 and 749 cm^{-1} signified the presence of R-CH group [40]. Some other significant bands appeared after bioreduction in the FTIR spectra were at 2,854 cm^{-1} and 1,737 cm^{-1} corresponding to the aromatic C–H anti-symmetric stretching vibration [41] and C=O stretching vibration [37], respectively. These findings supported the results of Goel et al. [42], who reported that proteins can bind to nanoparticles either through amino groups or cysteine residue or through electrostatic attraction. Furthermore, Ahmad et al. [43] and Xie et al. [44] reported that protein can interact with AuNPs either through free amine, carboxyl or phosphate groups to stabilize them. Thus, it can be concluded that the AuNPs are stabilized by surface bound protein molecules that also prevent aggregation. In addition to that microscopic fungi can generate extracellular AuNPs by a process involving NADH-reductase [45, 46].

Atomic force microscopy

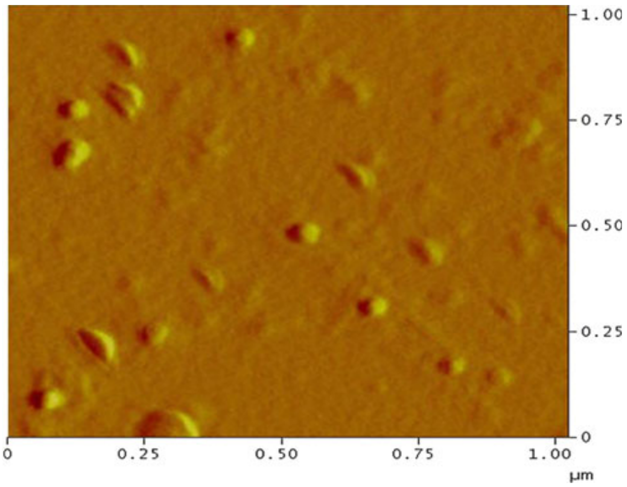

Fig. 6 Tapping Mode AFM phase image presented as two-dimensional graphics showing the clean spherical and triangular shaped gold nanoparticles

Results obtained from AFM study (Fig. 6) represent a clear concept regarding shape as shown in the picture. Majority of the particles were spherical and triangular in shape, well distributed without aggregation and monodispersity was an important characteristic of nanoparticles. The sizes of the AuNPs were found to be similar with the data collected from the DLS study (Fig. 2) and the variation in size of nanoparticles were commonly found during biological synthesis.

TEM image of gold nanoparticles

Transmission electron microscopic (TEM) image shown in Fig. 7 recorded different sizes of AuNPs which arose from the bio-reduction of chloroauric acid by culture filtrate at room temperature (37 °C) for 24 h. These observations revealed that spherical, triangular as well as hexagonal structures of the AuNPs were formed in the reaction solution. The diameters of these AuNPs were measured and

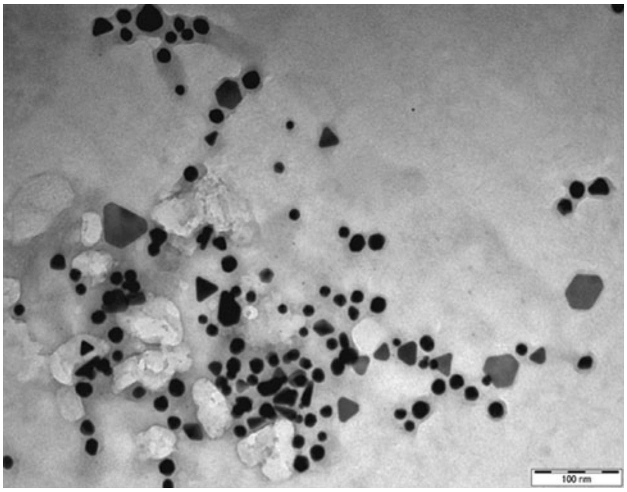

Fig. 7 Transmission electron micrograph of gold nanoparticles after bio-reduction of chloroauric acid

the size was in the range of 2–30 nm. The average diameter of these AuNPs was of 12 ± 5 nm.

Conclusion

In summary, we have demonstrated the biosynthesis of AuNPs using a phytopathogenic fungus *A. alternata*. The fungal reduction of chloroauric acid by *A. alternata* yielded AuNPs in the range of 2–30 nm. The present investigation suggests extracellular biosynthesis and stabilization of AuNPs by proteins present in the culture filtrate. The advantage of biosynthesis of nanoparticles using this protocol over other methods currently in use is that the nanoparticles are quite stable in solution. This study may therefore lead to the development of a simple bioprocess for the synthesis of AuNPs. It is expected that with further work on understanding the biochemistry of the fungal system and complete cataloging of biomolecules in the culture filtrate which participate in AuNPs formation, a green process using microorganisms as "nanofactories" may be developed to produce metal nanocrystals of increasingly complex and yet controllable structure.

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